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Characterization of newly established oral cancer cell lines derived from six squamous cell carcinoma and two mucoepidermoid carcinoma cells

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Abbreviations: ECM, extracellular matrix; EGFR, epidermal growth factor receptor; HPV, human papilloma virus; MEC, mucoepidermoid carcinoma; OSC, oral squamous cell carcinoma; SCC, squamous cell carcinoma; STR, short tandem repeats; YD, Yonsei University, School of Dentistry

Abstract

Since genetic abnormalities of human cancer are greatly geographically dependent, cultural and environmental backgrounds are thought to be closely related to the carcinogenic process. In the present study, eight human cell lines were established by culture from untreated carcinomas of the oral cancer, of which five were from primary oral squamous cell carcinomas (OSC), one from a mucoepidermoid carcinoma (MEC) and one each originating from metastatic OSC and MEC. All the studied tumor lines grew as monolayers, and showed: i) an epithelial origin by the presence of cytokeratin, and ii) tumorigenic potential in nude mice. Western blot analysis revealed i) over expression of EGFR in six of the cell lines ii) decreased expression of E-cadherin in six cell lines compared to normal human oral mucosa. A mutational analysis showed: point mutations of p53 at exon 7, with transversion, and at exon 8, with transition. These well-characterized human YD cell lines should serve as useful tools in the study of the molecular pathogenesis and biological characteristics of head and neck cancer cells, and in the future testing of new therapeutic reagents for oral cancer.

Keywords: carcinoma, mucoepideermoid; cadherins; carcinoma, squamous cell; cell line, tumor; mouth neoplasms; p53; receptor, epidermal growth factor

Introduction

There have been many studies on the establishment of cancer cell lines, they contribute to various basic research on cancer, as well as to diagnostic and therapeutic development. However, the establishment of an oral cancer cell line is especially difficult from the primary site, and only a few successful attempts have been reported (Easty *et al.,* 1981; Hu *et al.,* 1984; Roa *et al.,* 1985; Sacks *et al.,* 1988; Tatke *et al.,* 1995; Ji *et al.,* 2001). Most previously established cell lines have been reported from lymph node metastatic foci, or transplanted tumors in nude mice. The reasons for the low success rate of establishing cell lines from primary tumors are considered to be due to bacterial contamination when the tumor tissue was resected and the low cellular activity of primary tumors compared to that of the metastatic tumors. However, cell lines derived from recurrent tumors, or metastasis, appear to be less differentiated, less well organized in culture, and demonstrate a morphological divergence displaying fewer desmosomes and tonofilaments, than cells in primary tumor lines (Easty *et al.,* 1981). For these reasons, cell lines from primary tumors are required for understanding the biological characteristics of oral cancer. In addition, genetic abnormalities of human cancer are greatly geographically dependent, so cultural and environmental backgrounds are thought to be closely related to carcinogenic process. In this report, 8 carcinoma cell lines, derived from six untreated primary, and two metastatic, tumors of the head and neck, were established and characterized, and designated as the YD cell lines. Of these cell lines, two originated from metastatic lymph nodes. The molecular characteristics, including the frequency of 'high-risk' HPV infection and their proliferating activities, degree of differentiation, status of proto-oncogene EGFR, E-cadherin, and

mutation of tumor suppressor gene p53 were also studied.

Materials and Methods

Establishment of cell lines

The surgical specimens were obtained from the operation room suite, and transported to the laboratory in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand island, NY), supplemented with 10% fetal calf serum(FBS), 1×10^{-10} M cholera toxin, 0.4 mg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, and 2×10^{-11} M triiodothyronine. The cancer tissues obtained from the patients were treated with trypsin, and then the isolated cancer cells were grown with a feeder layer of mitomycin C-treated NIH 3T3 fibroblasts. The cells were fed with a mixture of DMEM and Ham's nutrient mixture F12 (Gibco BRL, Grand island, NY), at a 3:1 ratio, supplemented with 10% FBS, 1×10^{-10} M cholera toxin, 0.4 mg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, and 2×10^{-11} M triiodothyronine (T3). The cells were fed in an incubator at 37°C, with an atmosphere containing 5% CO_2. The characteristics of the patients from whom these cell lines were established are given in Table 1. Histopathology showed that five cell lines were from primary SCC and one from a primary MEC. One each originated from metastatic SCC and MEC.

Growth assay of the human OSC cell lines

Cells were plated at 1×10^4 cells/200 μl in each well of a 96 well plate. Every 24 h, 200 μl of the yellow MTT solution (0.05 mg/ml; Sigma Chemical Co., St. Louis, MO) was added to each well. The plates were covered with aluminum-foil, and then incubated at 37°C, in 5% CO_2 and 95% air, for 3 h. After incubation, purple formazan salt crystals were formed. The untransformed MTT that remained in the supernatant was removed by aspiration. The formed formazan crystals were dissolved by adding 150 μl of dimethylsulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO). The plates were then placed into an ELISA reader (Benchmark Microplate Reader, Bio-Rad, Herclues, CA), and the optical density of the plates read at 570 nm to measure the maximal absorbance of the solubilized formazan product. For each time period, the mean optical density value was calculated. After establishing the growth curves, the population doubling times of the established cell lines were estimated from the exponential growth phase.

DNA profiles

Genomic DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. For DNA-finger printing analysis, DNA was amplified by PCR using short tandem repeats (STR) markers. Eight cell culture samples were genotyped using PowerPlex16 (Promega, Medison, WI). Genotypes of 15 autosomal STRs (D3S1358, TH01, D21S11, D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D, vWA, D8S1179, TPOX and FGA) were determined in eight cell culture samples. Additional genotypes of 5 X-STRs (GATA172D05, HPRTB, DXS8377, DXS101, HumARA) and 9 Y-STRs (DYS19, DYS385a/b, DYS389-I, DYS389-II, DYS390, DYS391 and DYS393) were determined in YD-10B by PCR (Shin *et al.,* 2004). The PCR products were analyzed by capillary eletrophoresis using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

In vivo tumorigenicity

To investigate the *in vivo* tumorigenicity of the YD cell lines, $3\text{-}5 \times 10^6$ cells, mixed in DMEM, were injected subcutaneously into 4-6 week old male athymic nude mice (nu/nu; BALB/C), and the tumor formation was examined daily. When the mass had grown more than 1 cm, it was surgically removed, fixed in a 10% formalin solution, and paraffin-embedded, for histological evaluation.

Flow cytometric DNA measurement

The cells were washed twice with PBS, resuspended in 500 μl of ice-cold ethanol and maintained at 4°C for at least 30 min. Prior to analysis, the cells were washed again with ice-cold PBS, resuspended in 500 μl of ice-cold PBS and treated with 0.1 mg/ml RNase A (Sigma Chemical Co., St. Louis, MO) at 37°C for 30 min. Propidium iodide (PI; Sigma Chemical Co., St. Louis, MO), at a final concentration of 50 μg/ml, was then added to the cell suspensions. After incubation on ice for 30 min, the cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with an Argon laser, at 488 nm. The percentages of cells in the G0-G1, S, and G2-M phases were estimated under the assumption of a Gaussian distribution. The multicycle mode FIT 2.0 cell cycle software (version 2.0, Verity Software House Inc., Topsham, ME) was used to calculate the fraction of cells in the G0-G1 (represented by the first peak on the histogram), S (between the first peak and second peak), and G2-M (the second peak) phases. The protocol was repeated in triplicate for each group, and the means

and standard deviations determined.

Immunohistochemistry

For immunohistochemical staining, the paraffin-embedded tissues from both surgical specimens and the culture tissues were used. After deparaffinization and rehydration, the endogenous peroxidase was blocked with 3% H_2O_2 in methanol, and the sections incubated with normal horse serum at room temperature. Subsequently, a primary antibody, AE1/3 (DAKO, Glostrup, Denmark) or Vimentin (DAKO, Glostrup, Denmark), was applied overnight at room temperature to detect cytokeratins. Slides were then incubated with biotin-labeled horse anti-mouse/anti-rabbit IgG (Vector Lab., Burlingame, CA) at room temperature for 30 min. Subsequently, the slides were incubated, with horseradish peroxidase streptavidin, at room temperature for 30 min. 3,3-diaminobenzidine tetrachloride was used for visualization, and slides were counterstained with Meyer's hematoxylin.

Immunofluorescence studies

Cells were seeded on eight-chambered cover glasses (Nunc Inc., Naperville, IL) at 2×10^4 cells/chamber. Slides were fixed with acetone at -20°C for 10 min, then incubated with primary mouse monoclonal antibodies directed either against vimentin at 1/100 dilution (DAKO, Glostrup, Denmark) or cytokeratin at 1/50 (DAKO, Glostrup, Denmark) or AE1/AE3 (DAKO, Glostrup, Denmark) for 60 min. After washing, Alexa 594 donkey anti-sheep IgG conjugate (Molecular Probes Inc., Eugene, OR) were then added for 60 min and fixed again with ice-cold methanol for 10 min. All stained cells were stored under FluoroGuard antifade mounting reagent (Bio-Rad, Herclues, CA) and viewed by fluorescence microscopy.

Western blot analysis

Western blot analyses were performed as previously described (Yook *et al.,* 1998) using anti-p53 antibody (Pab 1801, Oncogene Sciences, Cambridge, MA), anti mouse E-cadherin monoclonal antibody (clone 36, BD Bioscience, San Diego, CA) and anti-EGFR antibody (clone E-30, Biogenex Co, San Ramo, CA) from the cell lysate. All the lanes contained equal amounts of protein, as determined by the Bradford method. After probing with each of the respective antibodies, the membranes were visualized by enhanced chemiluminescence.

Determination of HPV infection

A polymerase chain reaction (PCR) method was used for the detection of human papillomavirus (HPV) genotypes using general primer sets (Mork *et al.,* 2001). Two pairs of general primers were selected from the conserved L1 open reading frame, with the use of these primers in the PCR enabling the detection of the HPV genotypes HPV-1a, -6, -8, -11, -13, -16, -18, -30, -31, -32 and -33. The L1 general primers sequences were as follows: GP5 5' TTTGTTACTGTGGTAGATAC, GP6 5'GAAAAATAA-ACTGTAAATCA. The viral gene was amplified with DNA extracted from the OSC cell lines, using the L1 general primers. Caski (HPV16), HeLa (HPV18), and HT3 cell lines were used as positive and negative controls, respectively.

Mutation Screening in p53

DNA was extracted from the harvested culture cells using the phenol-chloroform method. PCR-SSCP analysis was performed to screen for p53 gene mutations in the OSC cell lines. The p53 exons 5-9 were each amplified with 100ng genomic DNA, 20 pmol of each primer, 2 umol dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$ and 0.2 U T*aq* polymerase (Perkin-Elmer, Boston, MA). The conditions used for PCR were: 30 cycles each of, denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and extension for 1 min at 72°C, in an automated thermal cycler (Perkin-Elmer, Boston, MA). The p53 primer sequences and PCR fragment sizes were as Table 1. SSCP was performed on 1 μl of PCR product diluted with 9 μl loading buffer (95% formamide, 0.25% bromophenol blue, 0.25% xylencyanol). Samples were denatured at 95°C for 5 min and immediately chilled on ice. Generally, 7 μl samples were applied to a 20% polyacrylamide gel and run at 100-150 V for 3-8 h. For optimal conditions, electrophoresis was done at 4°, 14.5° and 25°C using several PCR products in which mutations on each exon were confirmed by sequencing. Then Gels were then stained using the DNA Silver Staining Kit (Bioneer, Korea).

DNA sequencing analysis

The PCR products were cloned, using the pGEM T-easy vector system (Promega, Madison, WI), and subsequently sequenced using an automated DNA sequencer (Autoassembler 1.0, Perkin Elmer, Boston, MA). The HT3 (codon 245 of exon 7: GGC→ GTC) and Caski cell lines (wild type p53 gene) were used as positive and negative controls, respectively.

Results

Establishment of YD cells

Eight carcinoma cell lines, from surgical specimens

of oral carcinoma specimens, designated as the YD cell lines, were established, of which 3 were from tongue (YD-8, -10B, and -15) and 2 from lower gingiva (YD-17 and -38). The YD-15M and YD-17M were originated from metastatic lymph nodes and YD-9 from buccal cheek. Histopathologically, all tumors, except two, were classified as SCC. The clinicopathological findings of 6 patients were summarized in Table 1. Within the first 2 weeks of culture, the tumor cell clusters adhered to the surface of the dish, and gradually formed cell colonies. Once the tumor cells adapted to the *in vitro* conditions, they proceeded to grow rapidly. When the tumor cells had grown to semiconfluence in the dish, the first subculture was performed by transferring them to 25 cm^2 flasks (Nunc). Thereafter, subcultures were performed periodically in a solution of 0.2% trypsin and 0.1% ethylenediaminetetraacetic acid. All of the cell lines have been maintained for more than 2 yrs, passaged over 100 times, and continue to grow in a monolayer (Table 2).

DNA profiles

In order to confirm sample sources and assess the possibility of sample switching or contamination, eight cell culture samples were genotyped using PowerPlex16. Genotypes of 15 autosomal STRs (D3S1358, TH01, D21S11, D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D, vWA, D8S1179, TPOX and FGA) were determined in eight cell culture samples. The genotyping results of eight cell culture samples are unique and unrelated (Table 3A). Aadditional genotypes of 5 X-STRs (GATA-172D05, HPRTB, DXS8377, DXS101, HumARA) and 9 Y-STRs (DYS19, DYS385a/b, DYS389-I, DYS389-II, DYS390, DYS391 and DYS393) were determined in YD10-B which showed three banded allele pattern in vWA locus. At all 5 X-STR and 9 Y-STR loci, YD10-B samples displayed only one peak (Table 3B). These results help exclude the possibility of cross contamination among the cell lines.

Table 1. Clinical and pathological findings of established oral cancer cell lines.

Cell line	Age/Sex	Primary site	Pathologic diagnosis	Pathological nodal status
YD-8	46/F	Tongue	SCC, MD	
YD-9	56/M	Buccal cheek	SCC, MD	
YD-10B	67/M	Tongue	SCC, MD	
YD-15	39/M	Tongue	MEC, HG	5/17 left I 2/7, II 3/10
YD-15M		Lymph node	Metastatic	
YD-17	66/M	Lower gingiva	SCC, PD	6/41 Right I 1/10, II 5/15,
YD-17M		Lymph node	Metastatic	III 0/12, Left 0/4
YD-38	67/M	Lower gingiva	SCC, MD	

SCC, squamous cell carcinoma; MEC, mucoepidermoid carcinoma; MD, moderately differentiated; PD, poorly differentiated; HG, high grade

Table 2. Properties of established oral cancer cell lines.

Cell line	Doubling time	Growth characteristics	Prior chemotherapy	Passage	Cell cycle distribution				Tumourigenicity
					G0/G1 (%)	S (%)	G2/M (%)	DNA index	
YD-8	29.3	Adherent	None	>100	39.5	10.9	49.6	1.92	0/5
YD-9	33.4	Adherent	None	>142	59.2	22.5	18.3	1.91	0/5
YD-10B	25.3	Adherent	None	>137	42.8	19.0	38.2	1.91	5/5
YD-15	19.8	Adherent	None	>198	47.0	16.6	36.4	1.84	0/5
YD-15M	20.8	Adherent	None	>226	35.5	20.3	44.2	1.91	1/5
YD-17	29.2	Adherent	None	>196	51.9	16.2	31.9	1.91	0/5
YD-17M	19.6	Adherent	None	>100	51.5	12.5	35.9	1.86	0/5
YD-38	21.6	Adherent	None	>185	52.1	26.3	21.6	1.92	0/5

Table 3A. Observed alleles of the 15 autosomal STR markers in the YD cell lines.

Locus	YD-8	YD-9	YD-10B	YD-15	YD-15M	YD-17	YD-17M	YD-38
D3S1358	15-16	15-16	17-17	15-15	15-15	14-15	14-15	16-16
TH01	7-9	6	6-9	6-9	6-9	9.3-9.3	9.3-9.3	6-9
D21S11	29-30	30	30-32	30-31.2	30-31.2	28-31	28-31	30-31
D18S51	15-16	13	14-14	14-14	14-14	14-16	14-16	14-14
Penta E	12-15	15-17	5-8	14-19	14-19	5-12	5-12	12-19
D5S818	12-12	10-12	11-13	12-12	12-12	12-12	12-12	14-14
D13S317	8-10	13	9-9	11-12	11-12	8-8	8-8	8-8
D7S820	10-13	11-12	11-11	12-13	12-13	10-11	10-11	10-11
D16S539	9-11	12-13	10-11	9-12	9-12	9-12	9-12	12-13
CSF1PO	10-10	10-12	12-13	10-12	10-12	10-12	10-12	12-12
Penta D	13-13	9	8-9	9-9	9-9	9-13	9-13	8-12
vWA	14-17	14-19	18-19-20	15-15	15-15	15-16	15-16	14-17
D8S1179	13-16	11-13	11-11	15-15	15-15	13-15	13-15	14-14
TPOX	8-11	8-9	8-8	9-9	9-9	8-11	8-11	8-8
FGA	24-24	25	23-23	23-23	23-23	24-24	24-24	22-25

Morphologic and biologic characteristics

Figure 1 shows the photomicrographs of the YD cells. All cells adhered tightly to the flask in a monolayer sheet. The appearance of the YD-8, YD-15, YD-15M, YD-17, YD-17M, and YD-38 cells were polygonal, whereas the YD-9 cells were slightly elongated and the YD-10B cells were round to oval shaped tumor cells. All the YD cell lines grew as monolayers, and their population doubling time ranged from 19.8 to 33.4 h. The average doubling time of the established carcinoma cell lines was 24.9 h, and the YD-15, YD-15M, YD-17M cell lines, derived from metastatic lymph nodes, showed faster doubling times (Table 2). A total of 1×10^5 YD cells, after more than 100th passages, were analyzed by flow cytometry, and the results are summarized in Table 2. All of the cell lines were aneuploid, with a DNA index of around 1.9.

Analysis of epithelial markers and epithelial junction molecules

To detect cytokeratin expression in the primary and metastatic tumors of the YD cell lines, immunohistochemical staining was performed for AE1/3, and cytokeratin was expressed in the cytoplasm of all YD cell lines (Figure 2). In postembryonic cells, cytokeratins are the epithelial counterpart of vimentin; therefore, we also examined the expession of vimentin. Double immunostaining of cells revealed that all of the YD cells expressed only cytokeratin (Figure 2). Another important characteristic that distinguishes epithelial from mesenchymal cells is the cell junctional complexes formed by homotypic cell adhesion molecules, principally the E-cadherin. Western blot analysis was performed to measure the level of E-cadherin in extract from all of the YD cell lines. E-cadherin was expressed in all YD cells except, but the expression was decreased compare to normal human oral mucosa (Figure 3A).

Table 3B. Observed alleles of the five X- and nine Y-linked STR markers in the YD-10B cell line.

Locus	Observed alleles
GATA172	10
HPRTB	12
DXS8377	57
DXS101	22
HumARA	23
DYS392	11
DYS389I	13
DYS19	15
DYS389II	29
DYS393	13
DYS391	10
DYS390	24
DYS385a/b	12-14

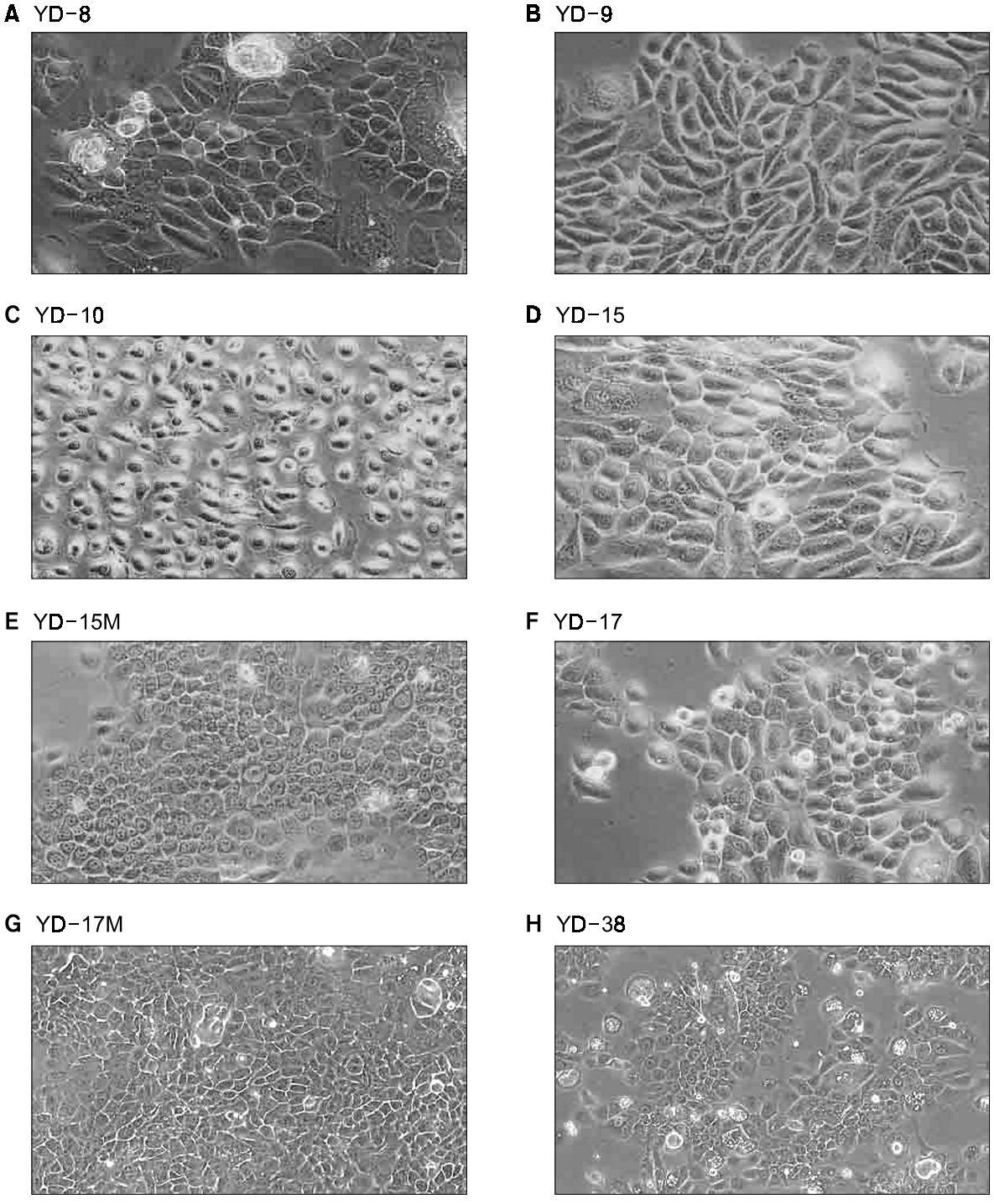

Figure 1. Microscopic features of established oral cancer cell lines. (A) YD-8 showing a sheet of polygonal cells scattered with dyskeratotic cells. (B) YD-9 showing slightly elliptical appearance of tumor cells. (C) YD-10B showing round to oval shaped tumor cells. (D) YD-15 showing relatively round, ovoid or angular shaped tumor cells. (E) YD-15M showing a sheet of oval shaped tumor cells. (F) YD-17 showing a sheet of polygonal cells. (G) YD-17M showing a sheet of polygonal cells similar to YD-17. (H) YD-38 showing a sheet of polygonal cells with irregular border.

Histological observation of transplanted tumors

To determine whether the YD cell lines were tumorigenic, 3-5 $\times$ 10^6 cells were subcutaneously injected into nude mice(five mice/cell line). As shown in Table 2, the YD-10B and -15M developed tumors. The incidence of transplantability by subcutaneous ino-

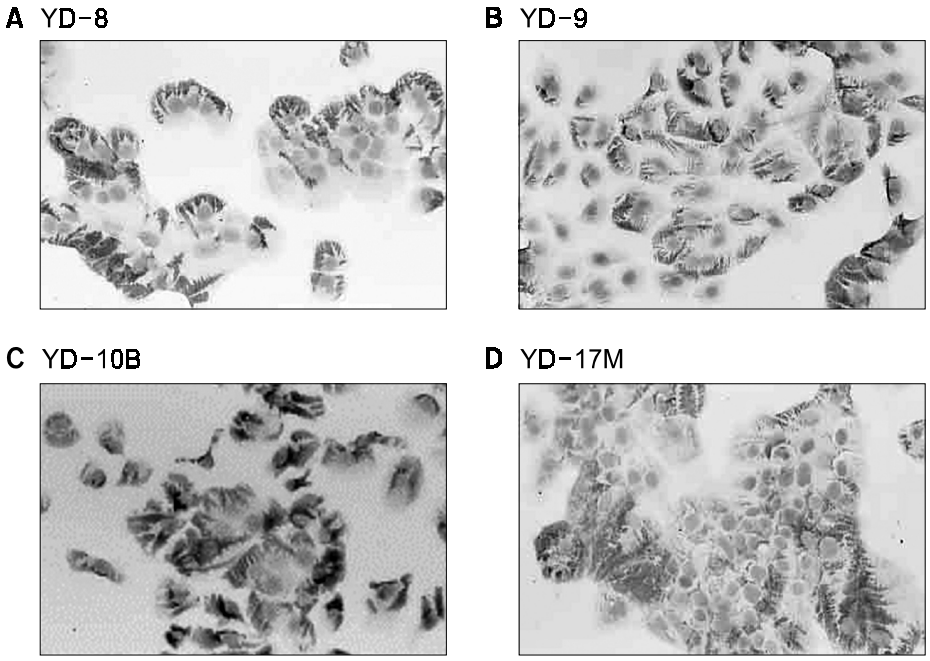

Figure 2. Expression of differentiation markers on the established cancer cell lines. Immunostaining for cytokeratins using the monoclonal antibody cocktail AE1/AE3 was performed. All YD cells showed AE1/3 immunoreactivity. AE1/3 is expressed in the cytoplasms of YD cell lines.

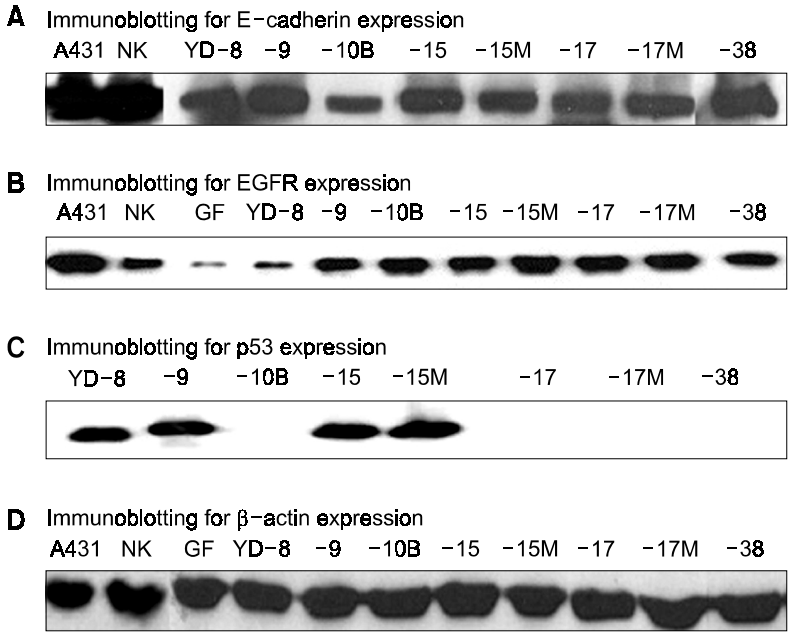

Figure 3. Expression of E-cadherin, EGFR, and p53 in the established oral cancer YD cell lines.(A) E-cadherin expression of established oral cancer cell lines. (B) EGFR expression of established oral cancer cell lines. Most of the cancer cell lines overexpressed EGFR except YD-8. (C) p53 expression of established oral cancer cell lines. (D) β-actin protein served as an internal control. A431, positive control; NK, normal keratinocytes, GF, gingival fibroblasts.

culation of cancer cells was 1/5 in YD-15M and 5/5 in YD-10B. Transplanted tumors were well circumscribed by surrounding fibrous tissue. No invasive growth was evident. The central portion of the transplanted tumors underwent severe ischemic necrosis. Transplanted tumor of YD-10B showed well-differentiated SCC and had similar morphology to their respective original tumor of the tongue. However, a transplanted of YD-15M showed a poorly differentiated SCC, this devoid of mucous cells or glandular differentiation (Figure 4).

Expression of proto-oncogenes EGFR

Figure 3(B) shows the expressions of EGFR in newly established YD cell lines, with most overexpressing EGFR, with the exception of the YD-8, compared to normal keratinocytes and gingival fibroblasts. The YD-8 cell line harbored a lower amount of the EGFR expression than normal keratinocytes.

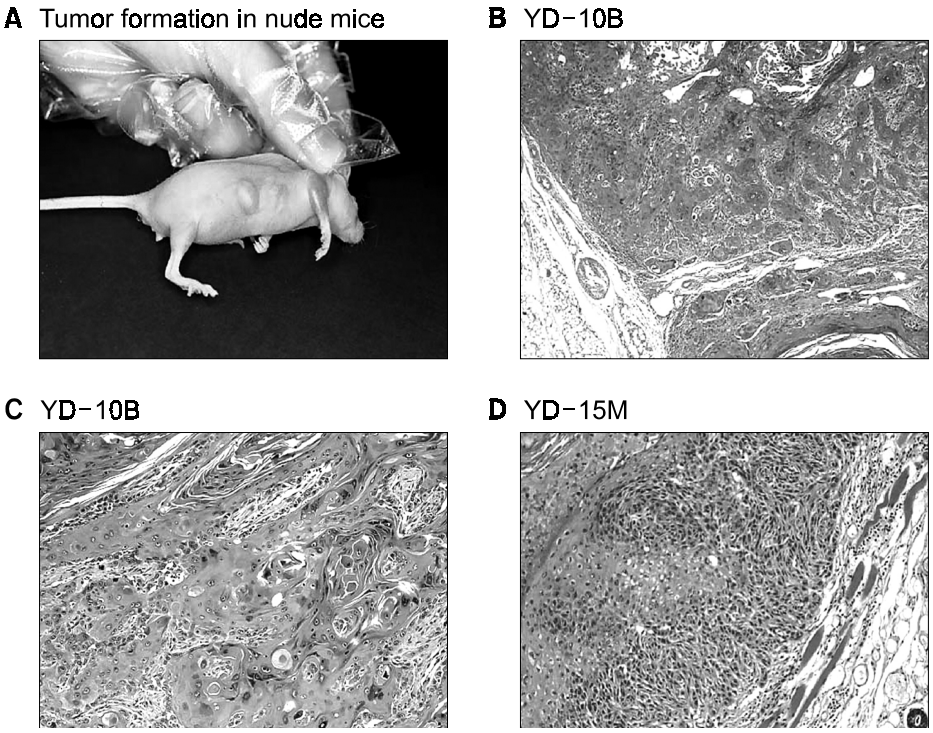

Figure 4. *In vivo* tumorigenecity in athymic nude mice. (A) Subcutaneous tumor formation in nude mice. The incidence of transplantability by subcutaneous inoculation of cancer cells was 1/5 in YD-15M and 5/5 in YD-10B. (B) Low magnifications (40 ×) of histopathologic findings of tumor of YD-10B in athymic nude mice showing a well differentiated squamous cell carcinoma. (C) High power of the tumor (100 ×) showing cellular pleomorphism and many mitotic figures. (D) A transplanted of YD-15M showed a poorly differentiated SCC, this devoid of mucous cells or glandular differentiation.

Mutational analysis in tumor suppressor genes p53

The presence of mutation of the p53 gene was examined by performing PCR-SSCP and by subsequent DNA sequencing of exons 4-9 of the p53 gene in the YD cell lines (Figure 5). Exon 7 showed point mutation in three cell lines (YD-10B, YD-15, and YD-15M) and exon 8 in one, the YD-8. Both the YD-15 and YD-15M cell lines revealed the same point mutation at codon 258 of exon 7, that is, the GAA sequence was changed to GCA, which resulted in an amino acid mutation of Glu to Ala. The YD-8 cell line also showed a point mutation at codon 273 of exon 8, the GGT sequence was changed to CAT, which was changed from Arg to His. The YD-10B cell line showed a frame shift mutation at codon 236 of exon 7, the TAC sequence was changed to TAA (Table 4). The level of p53 protein was analyzed by Western blot analysis. p53 protein was detected in the YD-8, YD-9, YD-15, and YD-15M (Figure 3C).

Determination of human papillomavirus (HPV) infection in the human OSC cell lines

'High-risk' HPVs have been found in OSC, and a large number of studies have examined the relationship between HPV infection and SCC of the oral region. The YD cell lines were examined for the presence of HPV DNA by PCR amplification, and all proved to be free of HPV DNA (Figure 6).

Discussion

Human cancer cell lines in cultures are widely used in cancer research, where experiments cannot be performed using tissue from *in vivo* specimens and are important tools in understanding the biology of human cancers. A number of tumor cell lines have been established from human cancers obtained from primary or xenografted tumors (Thomson *et al.,* 1995; Ku *et al.,* 2002). However, the establishment of SCC cell lines is considered to be difficult, and low success rates have been reported. For example, three esophageal carcinoma cell lines were established by one group using 100 specimens (Hu *et al.,* 1984), and another group reported the establishment of two head and neck lines from more than 100 specimens (Hauser *et al.,* 1985). In 1981, two teams reported the development of head and neck SCC cell lines. With explants outgrowth techniques, a series of cell lines, designated HN1-HN10, were derived from patients who had received radiotherapy, with or without chemotherapy (Easty *et al.,* 1981). The second group used feeder layer techniques, similar to the methods employed by us, to establish two carcinoma cell lines of the epidermis and four of the tongue (Rheinwald *et al.,* 1981). One patient with a tongue carcinoma had received prior radiation and chemotherapy, but three had not been treated. Using inactivated 3T3 mouse cell feeder layers developed these cell lines. Another research group established

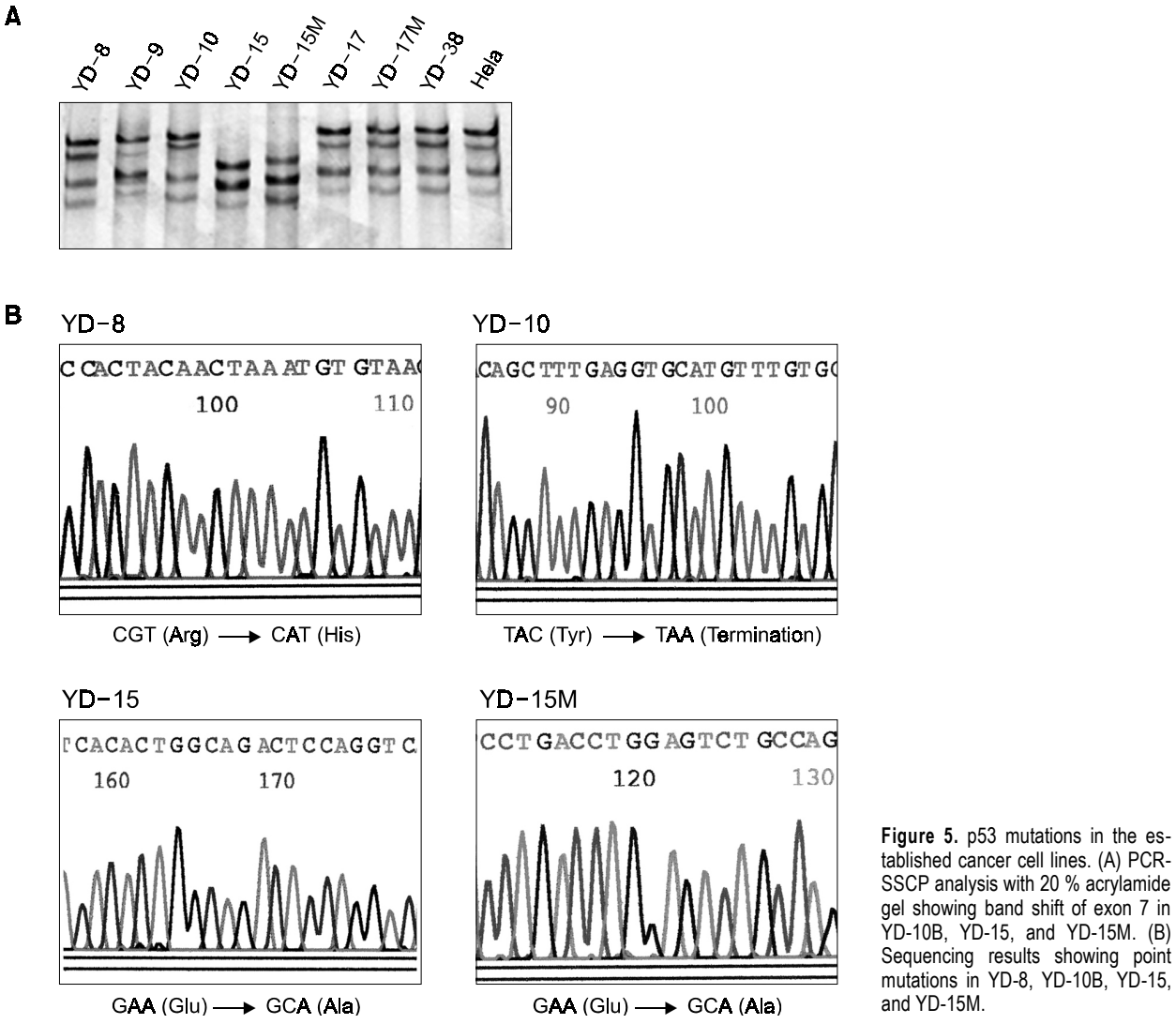

Figure 5. p53 mutations in the established cancer cell lines. (A) PCR-SSCP analysis with 20 % acrylamide gel showing band shift of exon 7 in YD-10B, YD-15, and YD-15M. (B) Sequencing results showing point mutations in YD-8, YD-10B, YD-15, and YD-15M.

Table 4. Mutational analysis of p53 in YD cell lines.

Gene	Cell line	Mutated codon (exon number)	Base change or deletion	Amino acid change
P53	YD-8	273(8)	CGT → CAT	Arg → His
	YD-10B	236(7)	TAC → TAA	Tyr → stop codon
	YD-15	258(7)	GAA → GCA	Glu → Ala
	YD-15M	258(7)	GAA → GCA	Glu → Ala

four new HNSCC cell lines, using outgrowth techniques, on tumors from patients who had received radiotherapy (Rupniak *et al.,* 1985). Thus, while the number of available HNSCC cell lines expands, there are still limited numbers in existence, and even fewer that are useful as models for the development and progression of this important type of tumor. Most of these cell lines were isolated more than 10 yrs ago, however, and have been passaged many times *in vitro* in uncontrolled growth conditions. Therefore, they could have lost their phenotypic heterogeneity. The use of carcinoma cell lines with a low passage number and newly established cell lines would solve this problem. Tumors are well known to be heterogeneous in a number of their parameters (Dexter *et al.,* 1982). Radiotherapy and chemotherapy, by eliminating sensitive cell populations, may alter a tumor's *in vivo* progression and cell heterogeneity (Nicolson *et al.,* 1984). Therefore, our interest was in establishing cell lines from tumors from Korean oral cancers, as the development of human cancers, including oral cancers, are known to be closely related to racial and cultural variables, and reference data from different cell lines are required from different

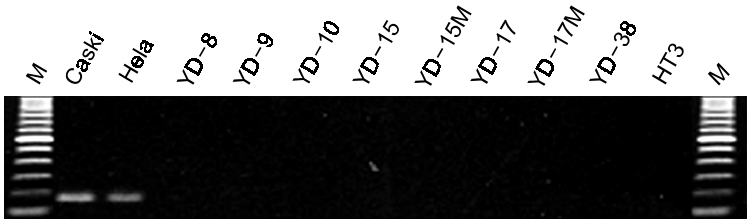

Figure 6. Detection of HPV DNA in the established cancer cellular lines. PCR amplification of tumor cell DNA showing no HPV DNA in established cancer cell lines. Caski and HeLa were used as positive control for HPV infection.

ethnic groups for untreated oral cancers as these are most likely to represent the original primary tumor. The eight new YD cell lines described here were established from untreated primary tumors. The success rate was at 18%. It is our belief that such cell lines are more likely to represent the initial tumor than lines derived from therapeutically treated patients.

OSC is the most common malignant neoplasm of the oral mucosa, accounting for more than 90% of all intraoral malignant tumors. Tobacco and alcohol use, viral infections, nutritional deficiency, and dietary habits have all been implicated in the etiology of head and neck cancer (Ahrendt *et al.,* 2000; Balaram *et al.,* 2002). Although the etiology of an OSC is unclear, substantial evidence indicates that the activation of proto-oncogenes, and the inactivation of tumor suppressor genes, underlies the disease development. To understand the molecular mechanisms underlying the development of OSC cell lines, the frequency of HPV infection and status of the proto-oncogene (EGFR), and tumor suppressors (p53) were investigated.

The HPV infection is also closely linked to benign and malignant oral lesions (Mork *et al.,* 2001). The involvement of the HPV infection in the development of oral cancer becomes evident from the frequent detection of 'high-risk' HPV DNA in OSC tissues and cell lines. Previous studies have reported the presence of HPV types 16 and 18 in oropharyngeal cancers specifically tonsillar cancers. In our study, we found that HPV16 presence was not significantly associated with our YD cell lines and tissues tested. Previous studies have similarly reported, HPV DNA from Korean fresh tumor tissue subjected to PCR amplification of the L1 or E7 viral region was undetectabe. Our findings suggest oral cancer was strongly associated with HPV16 presence in the Western but not Korean.

In our study, the p53 gene was point mutated in 33% and frame shift mutated in 11% of the YD cell lines and all of these mutations occurred in highly conserved regions. The majority of p53 mutations occur in the central conserved part of the protein or 'core domain' (residues 102-292), which is responsible for sequence specific DNA binding. In general, these mutations are of the missense type and lead to loss of DNA binding, which is believed to be critical for the biological activity of p53. In our study, codon 258 showed point mutation in two cell lines (YD-15 and YD-15M) and codon 273 in one, the YD-8. The YD-10B cell line showed a frame shift mutation at codon 236. These results raise questions over the functional significance of p53 gene mutations and this issue was investigated by analyzing p53 protein levels. Since the half-life of wild type p53 protein is several minutes, p53 protein levels in normal cells are relatively very low and generally undetectable by immunoblotting. However, abnormal p53 protein can be easily detected by immunoblotting because of the prolongation of its half-life. As shown in Figure 3, the over expression of p53 protein was found in all the p53-mutated YD cell lines, with the exception of one case with a nonsense mutation. However, one of the YD cell lines, not showing the p53 gene mutation, also positively expressed the p53 protein. There are several possible mechanisms, other than a point mutation, which can result in the over expression of the p53 protein (Yook *et al.,* 1998; Wong *et al.,* 2002). Genetic alternation in another region of the exon, such as the promoter or intron of the p53 gene, could result in a high expression of the wild type p53. However, 90% of mutations are clustered between exons 5 and 8 in four conserved regions of the p53 gene. Another possibility is the binding of the wild-type p53 protein to other proteins, such as MDM2 or E6 viral oncoprotein, stabilizes and inactivates as a result of the over expression (Barenes *et al.,* 1992; Momand *et al.,* 1992; Cho *et al.,* 2002; Seo *et al.,* 2004). The results of PCR-SSCP followed by direct DNA sequencing and immunoblotting demonstrated p53 gene mutations associated with abnormal accumulation of p53 protein in OSC cell lines. Since p53 is closely related to various proteins regulating the cell cycle, abnormal p53 function may cause accelerated cell proliferation leading to multicentric tumorgenesis and malignant tumor. However, 60% of multiple and malignant cases showed no mutations of the p53 gene. This suggests that p53 gene mutation is not a necessity for the development of multiple and malignant OSC.

To understand the possible role of proto-oncogenes in the development of the YD cell lines, the expression of EGFR was examined. Several studies

have demonstrated the over expression of EGFR in primary oral cancers and OSC cell lines (Frank *et al.,* 1991; Satini *et al.,* 1991; Shirasuna *et al.,* 1991). Our results showed the over expression of EGFR in newly established YD cell lines. Interestingly, YD-8 cell line showed much lower expression of EGFR compared to normal oral keratinocyte. Ludes-Mayers *et al.,* (1996) reported that p53 mutations might directly contribute to the gain of some growth promoting functions (gain of function phenotype), rather than represent the mere loss of the wild-type p53 function. Mutant p53 proteins are usually present at high levels in cancer cells, which may lead to the strong and continuous activation of expression in growth related genes, with oncogenic consequences. This observation gives rise to the possibility, that in cancer cells expressing high levels of mutant p53, the mutant protein may transactivate the promoter of the gene expressing human EGFR, resulting in more aggressive growth of these cells. The significance of the wild type p53-mediated transactivation of the EGFR promoter remains unclear. In our study that the YD-8 of p53 mutant cancer cells express high levels of mutant p53 but not EGFR is not likely to occur in p53 mutated cell line. Although our interest was in finding whether the EGFR was amplified dependently or independently of the mutant p53 protein, the details at present are indistinct.

Expression of the cell-cell adhesion molecule E-cadherin correlates with epithelial differentiation and and loss of E-cadherin may induce invasive growth of cancer cells (Batlle *et al.,* 2000; Ackland *et al.,* 2003). Our results showed that all YD cell line expressed E-cadherin. Morphological features of almost all cell lines tested showed polygonal, ovoid or elongated shaped cells representing epithelial differentiated, evidenced by cytokeratin expression.

In summary, eight newly established human OSC cell lines, with the histopathology of their primary tumors, *in vitro* growth characteristics, epithelial origin, and *in vivo* tumorigenicity have been reported. In addition, the status of their HPV infection, proto-oncogene (EGFR), tumor suppressor gene (p53), and E-cadherin are also reported. These eight cell lines will provide new permanent culture models for the study of the pathogenic mechanism, investigation their biological behavior, and to test new therapeutic reagents for oral cancer in the future.

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